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Increasing Numbers of Synaptic Puncta during Late-Phase LTP: N-Cadherin Is Synthesized, Recruited to Synaptic Sites, and Required for Potentiation

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Summary

It is an open question whether new synapses form during hippocampal LTP. Here, we show that late-phase LTP (L-LTP) is associated with a significant increase in numbers of synaptic puncta identified by synaptophysin and N-cadherin, an adhesion protein involved in synapse formation during development. During potentiation, protein levels of N-cadherin are significantly elevated and N-cadherin dimerization is enhanced. The increases in synaptic number and N-cadherin levels are dependent on cAMP-dependent protein kinase (PKA) and protein synthesis, both of which are also required for L-LTP. Blocking N-cadherin adhesion prevents the induction of L-LTP, but not the early-phase of LTP (E-LTP). Our data suggest that N-cadherin is synthesized during the induction of L-LTP and recruited to newly forming synapses. N-cadherin may play a critical role in L-LTP by holding nascent pre- and postsynaptic membranes in apposition, enabling incipient synapses to acquire function and contribute to potentiation.

Introduction

Long-term potentiation (LTP) is a strengthening of synaptic efficacy that can last for hours to days and may be a cellular mechanism for memory formation in the mammalian brain (Bliss and Collingridge, 1993). At the Schaffer collateral-CA1 synapse in hippocampus, LTP exhibits two distinct phases. An early phase (E-LTP) develops immediately following the posttetanic potentiation, lasts ~1 hr, and does not require new protein synthesis. E-LTP is thought to arise from rapid changes in the functional status of preexisting synapses, which may include conversion of synapses from a functionally inactive (silent) state to an active one (Isaac et al., 1995; Liao et al., 1995) and/or increases in the release probability of presynaptic vesicles (Liao et al., 1992; Bolshakov et al., 1997). In contrast, a late phase (L-LTP) develops more slowly, lasts for at least 10 hr and perhaps days, and can be blocked by inhibitors of gene transcription and new protein synthesis (Frey et al., 1988, 1993; Nguyen et al., 1994; Impey et al., 1996; Qi et al., 1996; Abel et al., 1997). These characteristics support proposals that L-LTP is based on new synapse formation, all the more compelling because comparable forms of long-lasting synaptic plasticity in invertebrates involve overt structural changes leading to new synapses (Bailey and Kandel, 1993). LTP in area CA1 has been correlated with growth of new dendritic spines (Engert and Bonhoeffer, 1999), an increase in the number of shaft synapses (Chang and Greenough, 1984), and an increase in the frequency of perforated synapses and multiple-synapse boutons (Buchs and Muller, 1996; Toni et al., 1999). Other studies, however, report that spine density remains unchanged (Andersen and Soleng, 1998) and that there are no changes in either total synapse number or frequency of multiple-synapse boutons (Sorra and Harris, 1998). Thus, whether new synapses accumulate in conjunction with long-lasting LTP remains controversial.

The establishment of pre- to postsynaptic membrane adhesion is one of the initial events in the construction of a synaptic junction and remains a fundamental component of the maintenance of synapses in maturity (Vaughn, 1989). Thus, it would be expected that synaptic adhesion proteins would play a central role in new synapse formation during long-lasting changes in synaptic function (Mayford et al., 1992; Schuster et al., 1996). While synaptic adhesion in the CNS is likely to be mediated by several families of proteins (Persohn et al., 1989; Takeichi, 1990; Einheber et al., 1996; Fannon and Colman, 1996; Fields and Itoh, 1996; Uchida et al., 1996; Song et al., 1999), there are compelling data suggesting that classic cadherins play a central role in synapse formation and targeting (see Shapiro and Colman, 1999, for review). Classic cadherins are localized to the synaptic junctional complex, where they bind homophilically to provide strong, calcium-dependent adhesive force across the synaptic cleft (Yamagata et al., 1995; Fannon and Colman, 1996; Uchida et al., 1996; Arndt et al., 1998; Benson and Tanaka, 1998; Huntley and Benson, 1999). In hippocampus, N-cadherin is one of the first proteins to be localized at developing synapses, becoming concentrated at synaptic sites and lost from other regions as they form (Benson and Tanaka, 1998). Beyond a static role in the adhesive maintenance of synaptic membrane apposition, cadherin function is also closely associated with synaptic physiology. For example, cadherins have been implicated in E-LTP (Tang et al., 1998; Manabe et al., 2000), although the mechanism through which they influence this form of rapid synaptic plasticity is unclear. Importantly, it was shown recently that strong depolarization of hippocampal neurons modifies the molecular configuration of synaptically localized N-cadherin to a state that represents enhanced synaptic adhesion, thus revealing that cadherin-mediated synaptic adhesion is dynamic and locally controlled by the activity of the very synapses at which N-cadherin is operative (Tanaka et al., 2000). There is, therefore, an intimate and dynamic relationship between the signaling function of the syn-

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apse and the cadherin-mediated adhesive scaffolding that contributes to maintaining synaptic architecture. This raises the possibility that long-lasting forms of synaptic plasticity could be achieved, at least in part, through activity-dependent modulation of cadherin adhesion or localization appropriate for changing synaptic morphology or for forming new synaptic junctions.

In this study, we address the question of whether synapses form during the protein synthesis–dependent late-phase of LTP and examine the role of N-cadherin in mediating this form of LTP in the context of synapse remodeling. We induce L-LTP selectively in hippocampal slices using a bath-applied, membrane-permeable, cAMP analog, an induction method that shares the same mechanism as electrically induced L-LTP but has the important advantage in that a greater fraction of the cells and their synapses is affected (Frey et al., 1993; Bolshakov et al., 1997). We then analyze by confocal microscopy large numbers of synaptic sites identified by the synaptic molecular markers N-cadherin and synaptophysin. Synaptophysin is a synaptic vesicle protein that labels presynaptic terminals. It is one of the first proteins found clustered at developing CNS synapses and has been used extensively at the light microscope level to detect developing and mature synapses (Navone et al., 1989; Fletcher et al., 1991; Ryan et al., 1993; Calhoun et al., 1996; Liu et al., 1999).

We find that L-LTP is accompanied by a protein synthesis–dependent increase in numbers of labeled synaptic puncta. The cAMP-induced increase in numbers of labeled synaptic puncta is associated with a significant rise in levels of N-cadherin protein, an indication that newly synthesized N-cadherin may be recruited to newly forming synaptic junctions or to preexisting ones that, prior to L-LTP, contained subthreshold levels of synaptophysin and N-cadherin. When we block N-cadherin-mediated adhesion during cAMP treatment or electrical stimulation sufficient for inducing E- and L-LTP, only the induction of L-LTP is abrogated, with no effect on normal synaptic transmission, E-LTP, or L-LTP once established. These data provide strong evidence that N-cadherin is synthesized and required for induction of late-phase LTP, during which time N-cadherin and synaptophysin accumulate at newly formed synaptic junctions or at preexisting ones that were subthreshold for detection prior to stimulation.

Results

L-LTP Is Accompanied by Increased Numbers of Synaptic Puncta

We induced L-LTP of Schaffer collateral-CA1 synapses by bath application of the membrane-permeable, cAMP analog Sp-cyclic adenosine 3′,5′-monophosphorothioate (Sp-cAMPS) to hippocampal slices for 15 min, which results in a persistent potentiation of the area CA1 field excitatory postsynaptic potential (EPSP). Figure 1A shows the time course of changes in the field EPSP initial slope, which reaches a maximum in 60–90 min. Synaptic puncta in Sp-cAMPS-potentiated slices ($n = 5$) and unstimulated control slices ($n = 12$) were visualized immunofluorescently with antibodies against synaptophysin or N-cadherin. High-resolution confocal microscopy (Figure 1B) and quantitative analyses (Figure 1C) of area CA1 show that potentiated slices have significantly greater numbers of N-cadherin puncta and synaptophysin puncta in comparison with unstimulated control slices ($p < 0.004$, unpaired two-tailed t test). Overlaying the N-cadherin and synaptophysin images from potentiated slices (Figure 2A) shows a codistribution pattern in which the majority of N-cadherin and synaptophysin puncta are just slightly offset from each other, resulting in a central region of overlap (yellow in the image) flanked on one side by N-cadherin labeling (green) and on the other by synaptophysin labeling (red) (Figure 2A, inset). This pattern indicates that, as expected, N-cadherin localizes to the synaptic junctional complex; the pattern is identical to that in the control sections and to previous descriptions (Fannon and Colman, 1996; Benson and Tanaka, 1998; Tang et al., 1998; Huntley and Benson, 1999; Tanaka et al., 2000). Similar to a previous report (Tang et al., 1998), most synaptophysin puncta in stratum radiatum appear to be associated with N-cadherin; however, there are some synaptophysin puncta that are not associated with N-cadherin antibody. We quantitatively assessed the degree to which N-cadherin and synaptophysin remained codistributed, and therefore "synaptic," in the potentiated slices relative to the control slices by using a Zeiss macro to determine the proportion of the total number of labeled pixels (green plus red) that were colocalized (yellow) and found the values to be similar (0.212 ± 0.016 versus 0.225 ± 0.025; values represent mean proportion [number of yellow pixels/number of red plus green pixels] $\pm$ SD, unpaired, two-tailed t test, $p = 0.44$).

Because no studies have yet demonstrated the synaptic localization of N-cadherin in hippocampal slices maintained in vitro, we used immunogold electron microscopy to verify the synaptic distribution of N-cadherin under the conditions imposed by our experiments. Figure 2B shows clusters of gold particles localized to the perimeter of pre- and postsynaptic membrane thickenings and the synaptic cleft in a potentiated slice, a pattern similar to previous studies in perfused tissue (Fannon and Colman, 1996; Uchida et al., 1996). Although it is impossible at present to determine whether or not the synapse shown is a potentiated one, this pattern of ultrastructural localization is representative of that found in both potentiated and control slices.

We determined that the increase in puncta numbers was not due to differential shrinkage of the neuropil (e.g., by loss of fine dendritic or glial processes), since in potentiated slices the area occupied by immunolabeling for the dendritic marker microtubule-associated protein 2 (MAP2) or for the astrocyte marker glial fibrillary acidic protein (GFAP) is not significantly different in comparison with unstimulated control slices ($p = 0.74$, MAP2; $p = 0.14$, GFAP, unpaired two-tailed t test).

While numbers of labeled synaptic puncta increase with L-LTP, morphometric analysis of immunolabeled puncta demonstrated that there are no significant differences between potentiated and control slices in the area of individual N-cadherin or synaptophysin puncta (Table 1). Moreover, puncta intensity levels did not differ significantly with either label between potentiated and control conditions (N-cadherin, $p = 0.42$; synaptophysin, $p = 0.20$, unpaired two-tailed t test).

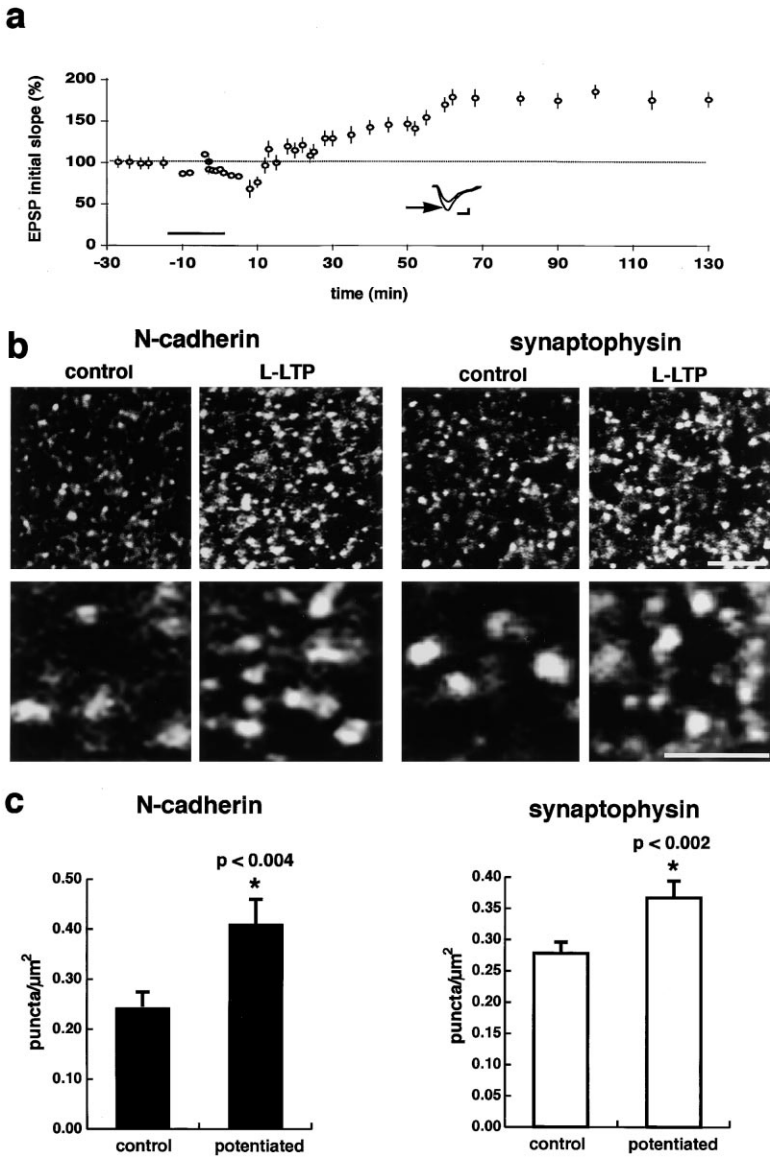

Figure 1. L-LTP Is Associated with Increased Number of N-Cadherin and Synaptophysin Puncta

(A) Bath application of Sp-cAMPS (50 μM; 15 min) produced a persistent potentiation of the extracellular field EPSP initial slope recorded from stratum radiatum, which reached maximum within 60–90 min (n = 9; error bars = SEM); bar indicates the duration of Sp-cAMPS application. Inset: representative EPSP traces were recorded before and 90 min after Sp-cAMPS application (arrow; calibration: 10 ms, 0.2 mV).

(B) Confocal microscope images through stratum radiatum of area CA1 showing increased numbers of N-cadherin and synaptophysin-immunolabeled puncta in potentiated (L-LTP) slices in comparison with unstimulated control slices. Scale bar: upper row = 5 μm; lower row = 2.5 μm.

(C) Quantitative analyses verified the number of labeled synaptic puncta in potentiated slices (n = 5) was significantly larger in comparison with unstimulated control slices (n = 12; mean ± SEM).

The Increase in Numbers of Synaptic Puncta during L-LTP Requires Synaptic Activity

Activation of the cAMP cascade can enhance spontaneous neurotransmitter release in area CA1 (Chavez-Noriega and Stevens, 1994), suggesting that ongoing synaptic activity during the establishment of L-LTP could contribute to the anatomical changes observed. To investigate this, slices (n = 4) were incubated in ionotropic glutamate receptor (GluR) antagonists (APV and CNQX, to block NMDA- and AMPA/kainate-type receptors, respectively) for 60 min, starting at the time of Sp-cAMPS exposure. The area CA1 field EPSP was greatly attenuated during the period of GluR blockade, although it was not blocked completely (data not shown), probably because of the presence of metabotropic glutamate receptors. After switching to normal Ringer's solution, the evoked field EPSP returned quickly to baseline—however, no potentiation emerged in these slices for up to 3 hr after washing out the GluR antagonists. In GluR antagonist-treated slices exposed to Sp-cAMPS, the numbers of N-cadherin and synaptophysin-labeled puncta are similar to those obtained from normal bath-maintained control slices or ones incubated in GluR antagonists alone (Figure 3; N-cadherin, $p = 0.6$; synaptophysin, $p = 0.9$, unpaired two-tailed t test). These data indicate that the Sp-cAMPS-induced changes in puncta number observed require ongoing synaptic activity and are therefore unlikely to result from unrelated effects of Sp-cAMPS upon general cellular metabolism.

The Increase in Numbers of Labeled Synaptic Puncta during L-LTP Requires PKA Activity and New Protein Synthesis

In slices removed and fixed 10 min following washout of Sp-cAMPS, and therefore prior to expression of L-LTP, the numbers of N-cadherin puncta are not significantly different than those obtained from unstimulated control slices (stimulated versus control: 0.30 ± 0.06 versus 0.24 ± 0.02 puncta/μm^2, mean ± SEM; $p = 0.9$).

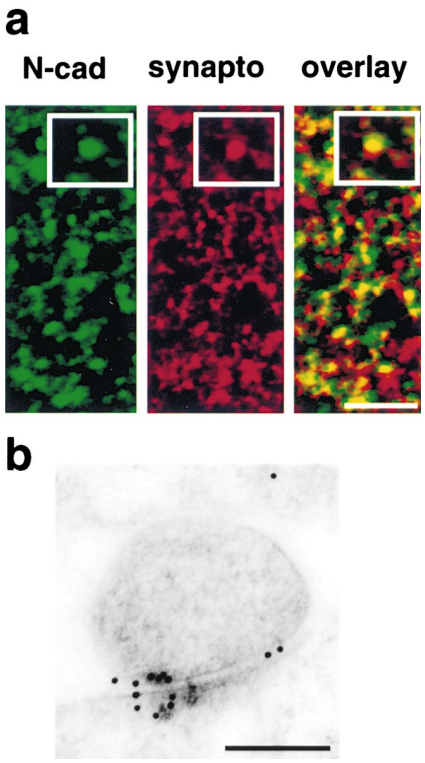

Figure 2. N-Cadherin Localizes to the Synaptic Junctional Complex in Potentiated Slices

(A) Confocal images from potentiated slices showing N-cadherin (green) and synaptophysin (red) immunolabeling separately and in an overlay where regions of colocalization appear yellow. Most N-cadherin and synaptophysin puncta codistribute in a partially overlapping pattern (shown in higher magnification inset), indicating that N-cadherin localizes to the synaptic juntional complex. Scale bar = 5 μm.

(B) Immunogold localization of N-cadherin from a potentiated slice demonstrating pre- and postsynaptic localization of N-cadherin at a synaptic junctional complex. Scale bar = 300 nm.

These data suggest that the anatomical and neurophysiological events develop contemporaneously and are causally related. Consistent with this, inhibitors of PKA and protein synthesis, which are known to block L-LTP (Frey et al., 1993), also block the increase in numbers of N-cadherin and synaptophysin puncta. Figure 4A shows that, as reported previously, Sp-cAMPS-induced potentiation is blocked by applying the competitive PKA inhibitor Rp-cAMPS or the protein synthesis inhibitor cyclohexamide prior to adding Sp-cAMPS. In these slices in which L-LTP is blocked, the numbers of N-cadherin (Figure 4B) and synaptophysin (Figure 4C) puncta are not significantly different in comparison with those obtained from control slices incubated in Rp-cAMPS or cyclohexamide alone. These data demonstrate that, like L-LTP, the increase in numbers of labeled synaptic puncta also requires PKA activity and new protein synthesis, suggesting a mechanistic relationship between increasing numbers of labeled synaptic puncta and expression of L-LTP.

Table 1. Sizes of N-Cadherin and Synaptophysin Puncta during L-LTP

Antibody	Condition	Area (μm^2)	n
N-cad	control	0.161 ± 0.007	8
	Sp-cAMPS	0.169 ± 0.004[a]	6
Synapt	control	0.179 ± 0.008	12
	Sp-cAMPS	0.178 ± 0.005[b]	5

Values are mean ± SEM; n = number of slices; [a]p = 0.65, [b]p = 0.97, t test between control and Sp-cAMPS.

cAMP Treatment Stimulates Rise in Levels of N-Cadherin Protein

That increases in numbers of N-cadherin puncta require protein synthesis suggests that Sp-cAMPS may stimulate an increase in the level of N-cadherin protein. We tested this using several approaches. First, immunoblots (Figure 5A, lanes 1 and 2) of area CA1 homogenates show a significant 27% increase in the level of N-cadherin protein in the potentiated slices in comparison with unstimulated control slices (Figure 5B, $p < 0.002$, t test). Second, N-cadherin immunoprecipitated from Sp-cAMPS-treated slices shows a similar significant rise in comparison with unstimulated slices (Figure 5A, lanes 3 and 4; $p < 0.003$, t test, $n = 2$). Third, we determined directly that Sp-cAMPS stimulated N-cadherin synthesis by a combination of metabolic labeling with ^{35}S-methionine followed by N-cadherin immunoprecipitation. Two faint bands, the uppermost of which is the expected size for N-cadherin (127 kDa), was observed in the unstimulated control slices (Figure 5C, lane 1). Fifteen minutes following Sp-cAMPS exposure, there was a substantial increase in the intensity of the same two bands and an additional band of slightly greater molecular weight (MW) (Figure 5C, lane 2), which increased further at 30 min (Figure 5C, lane 3). In accordance with previous studies, the highest MW band likely reflects the full-length N-cadherin polypeptide, the middle band, a slightly smaller polypeptide resulting from proteolytic cleavage at the N terminus, and the lowest MW band may correspond to a 110 kDa fragment resulting from C-terminal proteolytic cleavage or it may result from further trimming of sugars (Covault et al., 1991; Linnemann et al., 1994). No bands were detected in slices pretreated with cyclohexamide prior to Sp-cAMPS exposure (Figure 5C, lane 4). When the intensity of labeled bands was normalized to lane intensity, which also appears to increase nonspecifically, the increase in N-cadherin synthesis is substantial (5-fold) at 30 min (Figure 5D; t test, $p < 0.03$, $n = 2$). When taken together, these data show that Sp-cAMPS induces an increase in levels of N-cadherin.

Molecular Conformation of N-Cadherin during L-LTP

Strong stimulation of hippocampal neurons grown in culture modifies the molecular configuration of synaptically localized N-cadherin by inducing N-cadherin dimerization and imparting protease resistance (Tanaka et al., 2000)—indices that represent enhanced and stable adhesive interactions between membranes (Yoshida and Takeichi, 1982; Shirayoshi et al., 1986; Pokutta et al., 1994; Brieher et al., 1996; Tamura et al., 1998). We assessed the conformation of N-cadherin during L-LTP by immunoblots of the Triton-insoluble protein fractions

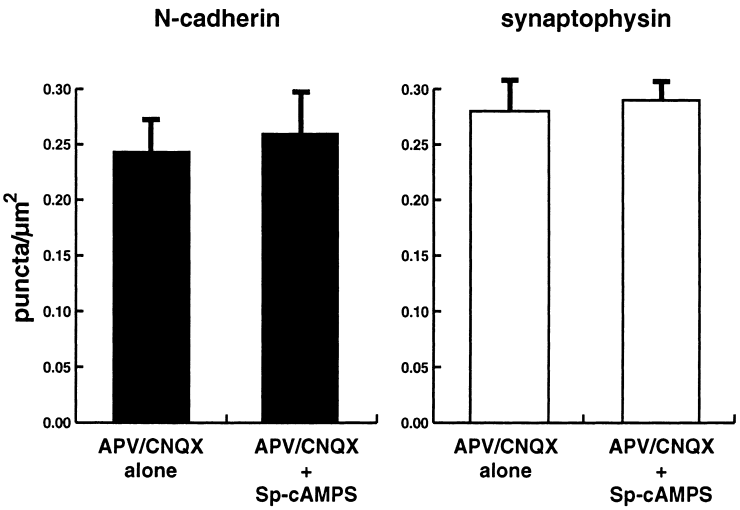

Figure 3. Ongoing GluR-Mediated Synaptic Activity Is Required for Sp-cAMPS-Induced Increase in Synaptic Puncta Number

Transiently exposing slices for 60 min to the NMDA receptor antagonist APV (50 μM) and the AMPA/kainate receptor antagonist CNQX (100 μM) inhibits L-LTP and prevents the increase in the numbers of N-cadherin puncta (left) and synaptophysin puncta (right) normally induced by exposure to Sp-cAMPS. Data are mean ± SEM.

from control and Sp-cAMPS-stimulated slices (Tanaka et al., 2000). We find that under either condition, there is a band of immunolabel of the size appropriate for the dimeric form (230 kDa) but that following Sp-cAMPS treatment, this band increases slightly, but significantly, in intensity (Figure 5E; t test, $p < 0.002$, $n = 2$). However,

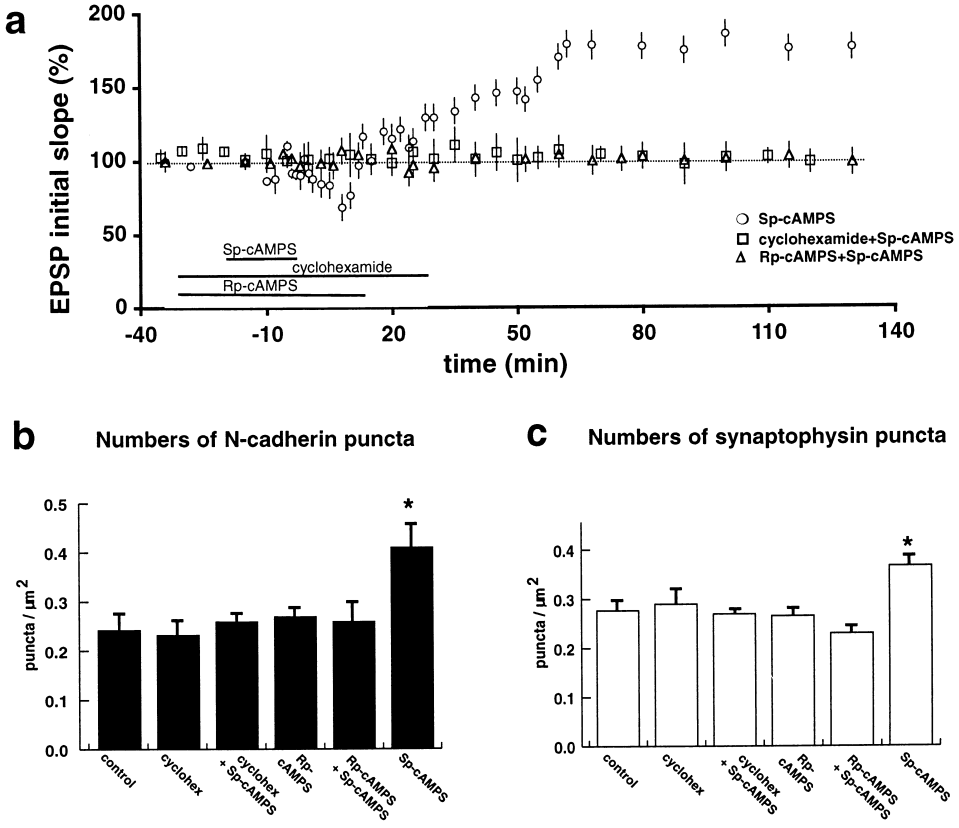

Figure 4. Increase In Numbers of Labeled Synaptic Puncta Requires PKA Activity and Protein Synthesis

(A) The potentiation in field EPSP slope induced by bath application of Sp-cAMPS (circles) is blocked by the protein synthesis inhibitor cyclohexamide (60 μM; squares) or the protein kinase A antagonist Rp-cAMPS (100 μM; triangles). Bars indicate periods of application of Sp-cAMPS, cyclohexamide, or Rp-cAMPS ($n = 4$ for each group; error bars = SEM).

(B and C) Inhibitors of protein synthesis and PKA that block L-LTP also block the Sp-cAMPS-induced increase in puncta numbers. Quantitative analyses demonstrate that in the presence of cyclohexamide or Rp-cAMPS, the number of N-cadherin (B) or synaptophysin (C) puncta in Sp-cAMPS-treated slices is not significantly different in comparison with that from slices treated with blockers alone or unstimulated control slices ($p > 0.1$) but is significantly less than the numbers observed in potentiated slices (asterisks, $p < 0.01$; $n = 4$ per condition, mean ± SEM).

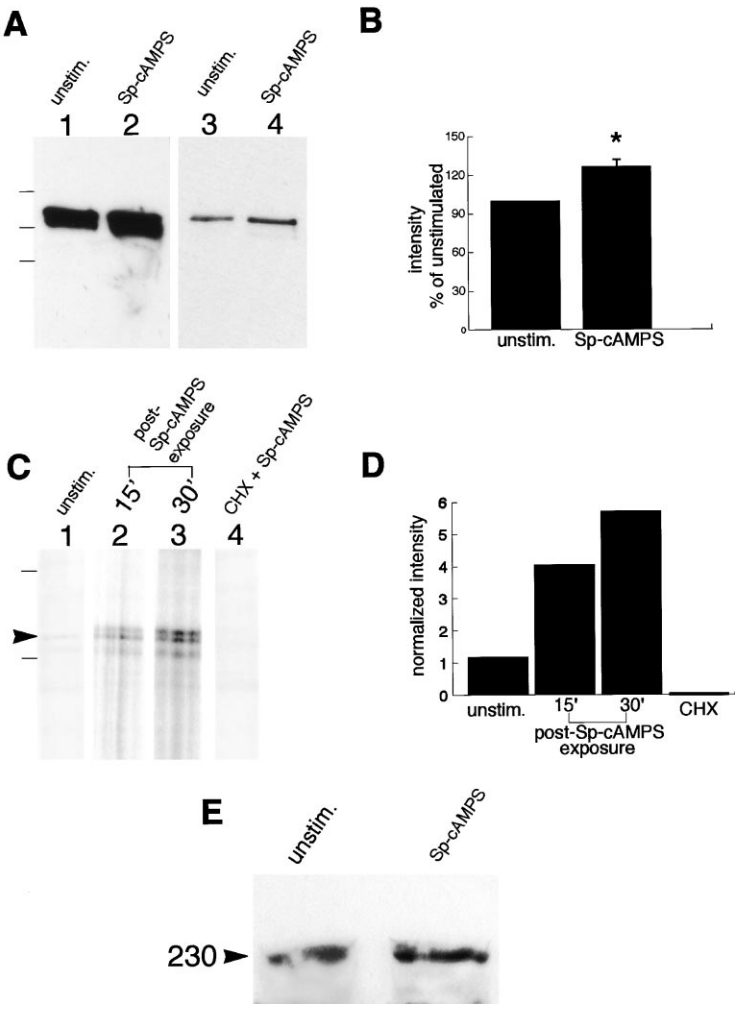

Figure 5. L-LTP Is Accompanied by an Increase in N-Cadherin Synthesis and Dimer Formation

(A) Immunoblot of unstimulated (lane 1) and Sp-cAMPS-potentiated (lane 2) slices. Protein fractions from the same samples were immunoprecipitated with an antibody against the EC1 domain of N-cadherin (lanes 3 and 4). Molecular weight markers: 205, 116, and 66 kDa.

(B) Quantitative analysis of immunoblots. There is a 27% ± 5.0% increase in N-cadherin level in potentiated slices relative to unstimulated control slices (mean ± SEM, n = 6 blots, $p < 0.002$).

(C) Metabolic labeling by ^{35}S-methionine incorporation followed by immunoprecipitation with the same N-cadherin antibody used in (A). Three bands (127 kDa, arrowhead) increase in intensity in slices removed 15 min (lane 2) and 30 min (lane 3) following Sp-cAMPS exposure in comparison with unstimulated control slices (lane 1). See text for explanation of bands. Treatment with cyclohexamide (CHX) prior to Sp-cAMPS exposure blocks N-cadherin synthesis (lane 4). Molecular weight markers: 205 and 116 kDa.

(D) Quantitative analysis of metabolic labeling experiment shown in (C). Band intensity was normalized to lane intensity.

(E) Analysis of the Triton-insoluble fraction reveals the strand dimer form of N-cadherin (230 kDa, arrowhead) in both unstimulated and potentiated slices.

under either condition, the vast majority of N-cadherin appears to exist in the monomeric form (data not shown). The modest enhancement in dimerization observed during L-LTP may reflect a "gentler" form of synaptic stimulation in comparison with the pronounced dimerization observed in cultured hippocampal neurons following strong depolarization with high concentrations of K^+ (Tanaka et al., 2000).

N-Cadherin Antibodies Block cAMP- or Electrically Induced L-LTP, but Do Not Affect E-LTP

To test whether N-cadherin-mediated adhesive interactions are required for L-LTP, we preincubated slices in one of two different affinity-purified N-cadherin antibodies that were raised against a portion of the ectodomain (EC1) containing the adhesive binding region. Antibody specificity was confirmed by Western blot, which showed a single band of ~127 kDa (Figure 6C) that could be competed out with purified N-cadherin EC1 protein. After transferring the antibody-incubated slices to normal bath solution and adding Sp-cAMPS, area CA1 field EPSPs were monitored for up to 3 hr, but in no case did potentiation develop (Figure 6A). We determined that the function-blocking antibodies adequately penetrated the tissue by subsectioning the slice at the end of the experiment and visualizing the bath-applied N-cadherin antibodies directly with fluorescently conjugated secondary antibodies (data not shown). Normal synaptic neurotransmission is not affected by antibody treatment, since stimulus-response curves of the antibody-treated slices are identical to those obtained from control slices (Figure 6B). Pretreatment of slices with preimmune sera did not block Sp-cAMPS-induced potentiation (Figure 6A). As an additional control, we preincubated slices with an N-cadherin antibody raised against the intracellular cytoplasmic portion of the molecule, which would therefore not be expected to have access to its intracellularly located epitopes. As expected, treatment with the intracellular N-cadherin antibody did not block Sp-cAMPS-induced potentiation (Figure 6A). We verified that the function-blocking antibodies were perturbing N-cadherin-mediated adhesion by an adhesion assay using mouse L cells stably transfected with N-cadherin, where, in the presence of Ca^{2+} and the function-blocking antibodies, N-cadherin-mediated adhesive aggregation is significantly diminished in comparison with N-cadherin-expressing L cells incubated with Ca^{2+} alone or ones incubated with Ca^{2+} and preimmune serum (Figure 7). Adding the function-

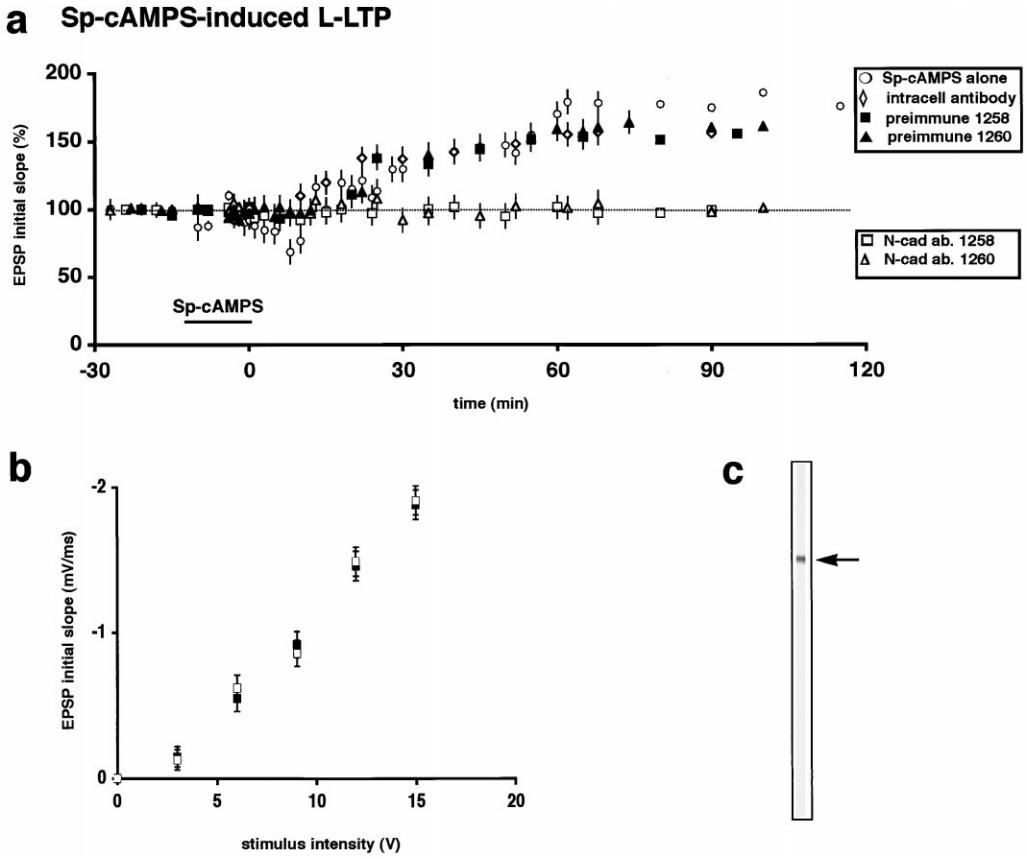

Figure 6. Treatment with N-Cadherin Antibodies Prevents Sp-cAMPS-Induced L-LTP

(A) Incubation of slices in one of two different N-cadherin antibodies (Ab 1258 [open squares] or Ab 1260 [open triangles]) blocks Sp-cAMPS-induced L-LTP ($n = 4$ for each antibody, error bars = SEM, $p < 0.05$, one-way ANOVA). Sp-cAMPS induced potentiation was unaffected by incubation with preimmune guinea pig sera (1258 [closed squares] or 1260 [closed triangles]) or with a control N-cadherin antibody recognizing an intracellular portion of the molecule (diamonds) ($n = 4$ for each group; error bars = SEM).

(B) Normal synaptic properties were unaffected by antibody treatment. Input-output curves indicating the relationship between stimulus intensity and field EPSP slope (mV/ms) show that slices incubated in Ab 1258 (closed squares) were not significantly different from normal bath-maintained control slices (open squares) ($n = 4$).

(C) Representative Western blot showing specificity of antibody 1260. Blot shows a single band of ~127 kDa (arrow) that is the approximate size of N-cadherin. Immunostaining could be competed out with purified N-cadherin EC1 protein but not with BSA.

blocking antibodies to hippocampal slices 1 hr after Sp-cAMPS exposure, and thus after L-LTP was established, fails to attenuate Sp-cAMPS-induced potentiation (data not shown). Taken together, these data indicate that the blocking antibodies have access to the adhesive domain only during the induction of L-LTP, presumably as new N-cadherin adhesive links are forming either during assembly of new synaptic junctions or as N-cadherin molecules are added to preexisting synapses. However, after L-LTP is established, the adhesive interface between cadherin–cadherin bonds may already be stably engaged and inaccessible.

The antibody-blocking effects on L-LTP are not an artifact of the particular chemically induced means of induction, since antibody pretreatment also blocks L-LTP induced by a tetanizing stimulation protocol sufficient for inducing L-LTP (Blitzer et al., 1995; Figure 8). Previous studies have established that electrically induced and Sp-cAMPS-induced L-LTP mutually occlude each other, indicating that the two induction protocols share the same mechanisms (Frey et al., 1993). Electrical stimulation protocols can induce both E- and L-LTP, in contrast to Sp-cAMPS in which only L-LTP is induced (Frey et al., 1993; Blitzer et al., 1995; Bolshakov et al., 1997). Figure 8 shows the immediate potentiation (E-LTP) in all slices following tetanizing stimulation, but only slices bathed in preimmune sera maintain L-LTP for at least 4 hr, when the experiments were terminated. In contrast, the immediate potentiation evident in the slices exposed to function-blocking antibodies attenuates significantly after 60 min (Figure 8) and remains attenuated for the duration of the experiment (4 hr). The incomplete return to baseline may reflect other mechanisms that contribute to the residual potentiation. These data demonstrate that N-cadherin-mediated adhesion is required for L-LTP, regardless of the means of induction. The gradual attenuation of LTP in the presence of blocking antibodies is consistent with the normal decay of E-LTP concurrently with a progressive blockade of N-cadherin adhesion during the transition to L-LTP, as pre- and postsynaptically apposed molecules attempt to link and lock across incipient synaptic junctions. The lack of a

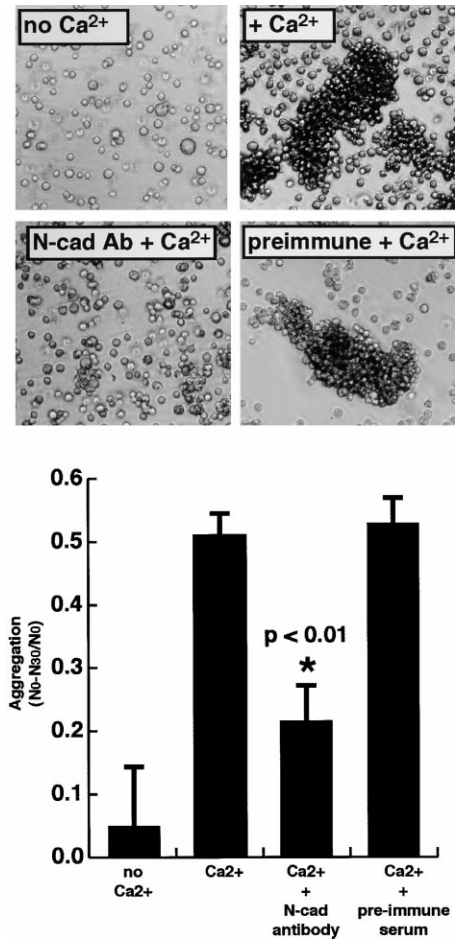

Figure 7. N-Cadherin Antibodies Significantly Attenuate Adhesive Aggregation of Mouse L Cells Stably Transfected with N-Cadherin

In the presence of Ca^{2+} and Ab 1260, the amount of aggregation is not significantly different than that of transfected L cells without Ca^{2+} but is significantly attenuated in comparison with transfected L cells incubated in Ca^{2+} alone or Ca^{2+} plus preimmune serum (one-way ANOVA). Data are mean ± SEM.

blocking effect on the immediate potentiation by N-cadherin antibodies contrasts with a previous study (Tang et al., 1998); these contrasting results may be due to differences in the induction protocols used and/or the use of different blocking antibodies. In this study, we specifically used an induction protocol that elicits both E- and L-LTP, whereas in the previous study, a higher frequency stimulation protocol for inducing E-LTP was used that may have transiently depleted extracellular Ca^{2+}, leading to destabilization of the cadherin ectodomain.

Increase in Numbers of Labeled Synaptic Puncta Persists in Presence of N-Cadherin Antibodies that Block L-LTP

While perturbing N-cadherin adhesion blocked the expression of L-LTP, we predicted that the function-blocking antibodies should not block the Sp-cAMPS-induced increase in puncta numbers, since Sp-cAMPS is membrane permeable and would be expected to activate its intracellular signaling cascade regardless of the presence of the extracellularly located antibodies. As anticipated, in slices in which L-LTP is blocked by preincubation with function-blocking N-cadherin antibodies, Sp-cAMPS stimulates a significant increase in the numbers of synaptophysin puncta (potentiated: 0.39 ± 0.01 puncta/μm^2, mean ± SEM; unstimulated control slices preincubated in preimmune sera: 0.29 ± 0.02; $p < 0.0001$, unpaired two-tailed t test, $n = 4$). It is not possible to determine whether the number of N-cadherin-labeled puncta also increases, since N-cadherin immunolocalization of extracellular epitopes is confounded by the presence of the N-cadherin-blocking antibody and intracellular antibodies to N-cadherin cross-react with the conserved cytoplasmic domain of other classic cadherins (Geiger et al., 1990). The block of synaptic potentiation without blocking the anatomical changes suggests that the blocking antibodies perturb a final, critical stage of functional synapse assembly required for L-LTP, either as new synapses form (Figure 9A) or as preexisting synapses undergo a molecular remodeling through the selective addition of N-cadherin and synaptophysin (Figure 9B). The function-blocking antibodies may prevent the formation of the adhesive interface between pre- and postsynaptically apposed cadherin molecules, thereby interfering with the appropriate spacing of the synaptic cleft or altering the area of the active zone (Figure 9C). It is equally possible that intracellular signaling by the cadherin-catenin complex is perturbed (Takeichi, 1990; Yap et al., 1997).

Discussion

We demonstrate here that the induction of L-LTP is accompanied by a significant increase in overall numbers of labeled synaptic puncta identified with molecular markers of synaptic junctions. Because we used an induction protocol that produces only L-LTP, but not E-LTP, we conclude that the increase in numbers of labeled puncta is associated specifically with the late-phase of LTP. Furthermore, we show that the increase requires PKA activity and new protein synthesis, both of which are known to be required for L-LTP, but not E-LTP (Huang et al., 1996). Additionally, our data provide novel insights into the molecular basis for synapse assembly or remodeling and L-LTP. We find that the bath-applied cAMP analog, which selectively induces L-LTP, stimulates contemporaneously a protein synthesis–dependent increase in levels of N-cadherin and an enhancement in N-cadherin dimerization, an index of enhanced adhesive force, indicating a specific role for N-cadherin and synaptic adhesion in the molecular cascade leading to long-lasting synaptic potentiation. Consistent with this, when we block N-cadherin-mediated adhesion with function-blocking antibodies, we prevent the induction of L-LTP but do not affect E-LTP or normal synaptic neurotransmission. Taken together, these data indicate that late-phase LTP requires N-cadherin adhesion as new synapses form or as preexisting synapses, which prior to L-LTP contained subthreshold levels of synaptophysin and N-cadherin, undergo a molecular remodeling.

Our data show clearly that the increase in numbers

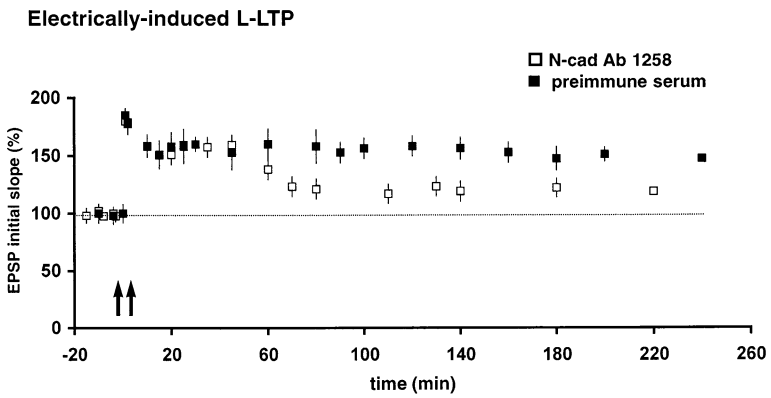

Figure 8. N-Cadherin Antibodies Attenuate L-LTP Induced by Electrical Stimulation without Affecting E-LTP

Tetanic stimulation (double arrows) was used to produce both E-LTP and L-LTP. Function-blocking antibodies did not block E-LTP but significantly attenuated potentiation ~60 min posttetanization (L-LTP), contemporaneous with the transition from E-LTP to L-LTP. In contrast, slices incubated in preimmune serum (closed squares) exhibited both E- and L-LTP; ($n = 4$; error bars = SEM, $p < 0.05$, one-way ANOVA).

of labeled synaptic puncta during L-LTP occurs in the absence of any prior E-LTP, indicating that the mechanisms responsible are separable from those that are triggered by, or directly associated with, E-LTP. This conclusion complements the results of Engert and Bonhoeffer (1999), who demonstrated a net increase in the formation of new dendritic spines only during long-lasting, but not short-lasting, LTP. Our data showing an increase in numbers of synaptophysin boutons may be an indication that such newly forming spines make functional synaptic contacts with new presynaptic boutons. In support of this, we find in the potentiated slices that the majority of N-cadherin puncta remain codistributed with synaptophysin puncta similar to control slices, implying that the new puncta visualized are also mostly synaptic (Fannon and Colman, 1996; Benson and Tanaka, 1998; Tang et al., 1998; Huntley and Benson, 1999; Tanaka et al., 2000). However, our data do not rule out the likelihood that molecular signaling associated with E-LTP, such as a synaptic tag (Frey and Morris, 1998), plays a role in directing subsequent molecular mechanisms that lead to synapse formation or remodeling during the transition to L-LTP. This would preserve the input specificity of LTP by the "capture" of synapse growth, signal transduction, and adhesion proteins in the vicinity of the tagged, preexisting synapses (Martin et al., 1997; Casadio et al., 1999), resulting in local synaptic proliferation or remodeling along those dendritic and axonal regions mutually engaged in synaptic potentiation (Engert and Bonhoeffer, 1999). Since classic cadherins display predominantly homophilic adhesive binding specificities (Takeichi, 1990; Yap et al., 1997), N-cadherin localization to both pre- and postsynaptic sides might guarantee input specificity at a molecular level during synapse formation by ensuring that only those incipient pre- and postsynaptic partners displaying like-cadherins would engage in synaptic contact (Fannon and Colman, 1996; Inoue and Sanes, 1997; Arndt et al., 1998; Benson and Tanaka, 1998; Huntley and Benson, 1999; Shapiro and Colman, 1999).

It has been argued on the basis of quantal analysis of unitary synaptic transmission between single CA3 and CA1 neurons that L-LTP is associated with an increase in numbers of functional release sites (Bolshakov et al., 1997). One interpretation of our data is that this is achieved through the formation of new pre- and postsynaptic specializations. This could occur through the splitting of preexisting boutons into two (or more) separate synaptic sites. Multiple-synapse boutons are normally rare in area CA1 (Schikorski and Stevens, 1997; Shepherd and Harris, 1998) and their formation is not a regular component of developmental synaptogenesis (Sorra et al., 1998). However, recent ultrastructural studies show an increased frequency of multiple-synapse boutons during LTP (Toni et al., 1999). Our data may therefore reflect in part the formation of multiple-synapse boutons. It has been observed in other paradigms of hippocampal synaptic plasticity that multiple-synapse boutons are larger in size in comparison with single-synapse boutons (Woolley et al., 1996). Because there is a positive correlation between bouton size and numbers of vesicles (Schikorski and Stevens, 1997; Shepherd and Harris, 1998), we looked for, but did not detect, differences between control and potentiated slices in the size of the synaptophysin puncta. This may be because the number of newly formed multiple-synapse boutons during L-LTP was greatly exceeded by the numbers of new synapses of the more prevalent, single-contact type and thus went undetected, or the level of resolution of the confocal microscope was insufficient for this type of analysis. There are other mechanisms that have been proposed to increase the number of functional synaptic sites during L-LTP that do not require the de novo assembly of synaptic junctions. Studies using FM-143 dye uptake by active presynaptic terminals in cultured hippocampal neurons before and after treatment with Sp-cAMPS suggest the existence of a population of preexisting but presynaptically silent boutons that are induced during L-LTP to an active state (Ma et al., 1999). It is equally possible that postsynaptically silent synapses—ones containing only NMDA-type glutamate receptors in the nonpotentiated state—are unmasked during potentiation by the insertion into the postsynaptic membrane of AMPA-type glutamate receptors (Malenka and Nicoll, 1999; Petralia et al., 1999; Shi et al., 1999). Our data do not address the contribution of either form of silent synapses to L-LTP, nor are they incompatible with these mechanisms occurring as well. However, if conversion of preexisting synapses from a silent to an active state were the only or predominant mechanism underlying L-LTP, then the numbers of boutons identified by synaptophysin labeling would not be significantly altered by potentiation, since it would be expected that preexisting but silent synapses would

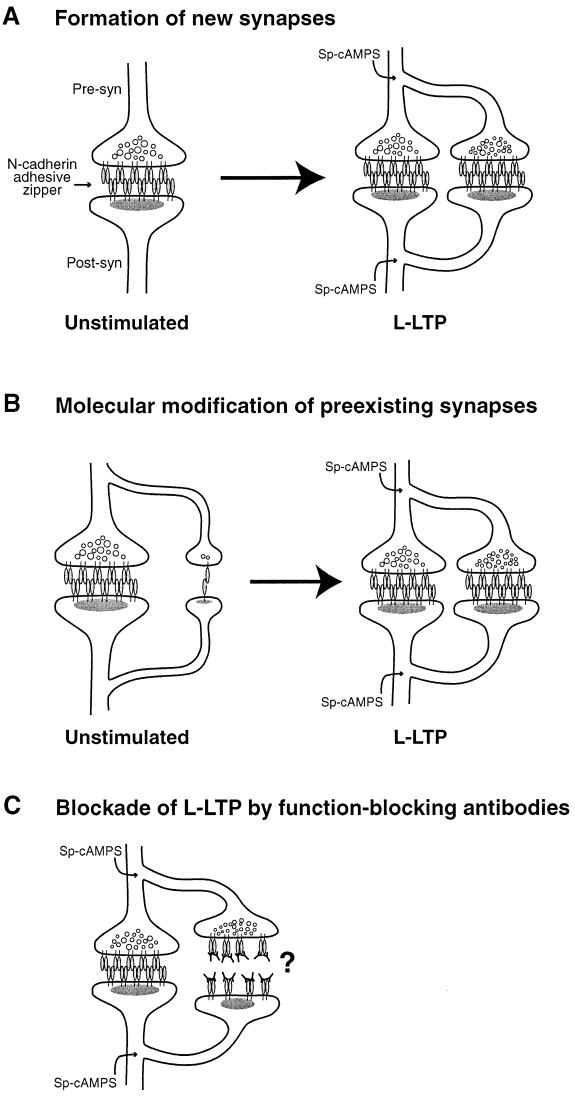

Figure 9. Speculative Mechanisms Leading to Increasing Numbers of Synaptic Puncta during L-LTP

Two possible models (A and B) to account for the increased puncta number during L-LTP. In both models, the membrane-permeable Sp-cAMPS activates an intracellular, cAMP-dependent signaling cascade that leads to a rise in N-cadherin protein levels. In the first model (A), N-cadherin and synaptophysin are recruited to new synapses that form de novo. Alternatively (B), some preexisting synapses may be subthreshold for detection in the unstimulated state (left), possibly because the synapses are too small and contain too few molecules to be detected by the markers used. However, during L-LTP these synapses become detectable through molecular remodeling involving an accumulation of N-cadherin and synaptophysin (right). Regardless of which mechanism (A or B) leads to L-LTP, potentiation is blocked in the presence of the function-blocking N-cadherin antibodies (C), possibly because the antibodies prevent the formation of the adhesive interface between pre- and postsynaptically apposed cadherin molecules, thus interfering with the appropriate spacing of the synaptic cleft or altering the area of the active zone (question mark). The increase in numbers of labeled synaptic puncta is still evident in L-LTP-blocked slices, presumably because Sp-cAMPS still activates its intracellular signal cascade both pre- and postsynaptically. For simplicity, the intracellular binding partners of the cadherins are not depicted.

remain identifiable by synaptophysin labeling of the clustered vesicles poised within the presynaptic boutons. This, however, is contrary to our observations of significantly increased numbers of synaptophysin boutons. Nevertheless, we cannot rule out an alternative explanation of our data that there exists a population of synapses that contain subthreshold levels of N-cadherin and synaptophysin in the unpotentiated state but undergo a molecular remodeling during L-LTP in which N-cadherin and synaptophysin are added, making these synapses detectable by the methods used (Figure 9B). This scenario would require mechanisms for recruiting both N-cadherin and synaptophysin selectively to such synapses, since our data show that there are no differences in the intensity of labeled puncta between unstimulated and potentiated slices, precluding a nonspecific addition of these molecular markers to all synapses.

The role of protein synthesis in hippocampal L-LTP and the identity of the proteins synthesized have remained elusive. In *Aplysia*, the cAMP-dependent PKA pathway, acting through the transcription factor CREB, stimulates the formation of new synaptic contacts during the transition from short-term to long-term memory storage (Bailey and Kandel, 1993). Our data reveal striking similarities in that the increase in numbers of labeled synapses in response to Sp-cAMPS-induced L-LTP was blocked by inhibitors of PKA and protein synthesis. Our finding that Sp-cAMPS stimulated a protein synthesis–dependent rise in levels of N-cadherin suggests that N-cadherin synthesis is a critical target of the cAMP-dependent PKA signaling cascade required for L-LTP. Like long-term facilitation in *Aplysia*, cAMP-dependent PKA signaling in hippocampal neurons activates the transcription factor CREB, which in turn regulates gene expression during L-LTP (Impey et al., 1996). It is not yet known if CREB regulates N-cadherin expression. Nevertheless, it is likely that the source of N-cadherin, as it progressively accumulates at synaptic sites during L-LTP, is from both new N-cadherin protein synthesis and from a locally recruitable pool of surface N-cadherin, one which is not tethered via the catenins to the actin cytoskeleton and therefore highly mobile (Tanaka et al., 2000).

The involvement of N-cadherin-mediated adhesion in L-LTP is striking in the context of new synapse formation or synapse remodeling. Cadherins are thought to play an important role in synapse formation during development by providing the adhesive scaffolding required for linking and maintaining pre- and postsynaptic membranes in apposition (Colman, 1997). Whether, during L-LTP, N-cadherin functions solely as a synaptic adhesive glue remains to be determined. Nevertheless, the selective blockade of L-LTP by antibodies whose adhesion-blocking properties were tested (Figure 7) supports the idea that cadherin-mediated synaptic adhesion is a critical molecular link between functional expression of L-LTP and the structural assembly of synaptic junctions upon which long-lasting synaptic plasticity may be based. It is unlikely that the antibody-blocking effects we observed were nonspecific. First, the antibodies prevented N-cadherin-mediated aggregation of L cells stably transfected with N-cadherin, suggesting that they function similarly by attenuating cadherin-mediated adhesion in the hippocampal slices. Second, two different

antibodies, both of which were raised against a portion of the molecule containing the adhesive binding domain, were equally effective at blocking L-LTP. Third, L-LTP was blocked by the antibodies regardless of whether it was elicited electrically or chemically. Fourth, the antibody effects on synaptic function were selective: only L-LTP was blocked—the antibodies had no effect on normal synaptic properties, nor did they affect the induction of E-LTP. Finally, L-LTP was unaffected by an N-cadherin antibody directed at an intracellular, and therefore inaccessible, portion of the molecule. N-cadherin antibodies could be disrupting the functional expression of L-LTP directly by sterically inhibiting or altering adhesion between N-cadherin extracellular domains sufficient for changing the spacing of the synaptic cleft or the area of the active zone (Figure 9C) or by disrupting an outside-in signaling pathway initiated by cadherin–cadherin contact. Since classic cadherins are linked to the actin cytoskeleton via the catenin proteins (Takeichi, 1990; Yap et al., 1997) and dynamic actin filaments are required for LTP (Krucker et al., 2000), it is possible that cadherins affect synaptic strength during LTP through a proactive role in changing spine morphology or motility (Geinisman et al., 1993; Segal, 1995; Fischer et al., 1998; Halpain et al., 1998; Engert and Bonhoeffer, 1999; Kirov and Harris, 1999; Kirov et al., 1999; Maletic-Savatic et al., 1999).

Strong depolarization of cultured hippocampal neurons changes the conformation of synaptic N-cadherin to the lateral strand dimer configuration, which, in turn, confers a pronounced resistance of N-cadherin to proteolytic degradation (Tanaka et al., 2000)—indices of strong adhesive activity. That N-cadherin bonds can display such a dynamic modulation of adhesive strength by the functional status of the very synapses at which they are localized (Tanaka et al., 2000), and perhaps as well by a variety of signaling pathways (Yap et al., 1997), suggests an ongoing and reversible modulation of adhesive strength. Our data support this view in that the strand dimer was detected in the control slices and was slightly enhanced in the potentiated ones, raising the possibility that under conditions of increasing synaptic efficacy, such as during LTP, synapses are stabilized by increased adhesive force during the initial phases of long-term memory encoding, followed subsequently by diminished adhesive force to promote synapse disassembly as the information encoded by such new circuitry is then transferred to other brain areas (Squire and Zola, 1997). It is likely that a broad array of adhesion molecules that utilize different adhesive mechanisms will be found to act coordinately to modify temporally and mechanistically distinct aspects of synaptic plasticity (Staubli et al., 1990; Luthl et al., 1994; Muller et al., 1996; Bahr et al., 1997), which underscores the dynamic relationship between the signaling function of synapses and the adhesive elements that build, maintain, and modify the structural scaffolding for signaling.

Experimental Procedures

Preparation of Hippocampal Slices and Electrophysiology

Hippocampal slices (350 μm thick) were prepared from 2- to 4-week-old Sprague–Dawley rats and continously perfused with Ringer's solution containing (in mM): NaCl, 125.0; KCl, 2.5; $MgSO_4$, 1.3; NaH_2PO_4, 1.0; $NaHCO_3$, 26.2; $CaCl_2$, 2.5; glucose, 11.0, bubbled with 95% O_2/5% CO_2, at 24°C during extracellular recordings (electrode solution: 3 mM NaCl). Slices were maintained for 1–2 hr prior to establishment of a baseline (30–45 min) of field EPSPs recorded from stratum radiatum in area CA1, evoked by stimulation of the Schaffer collateral-commissural afferents every 30 s with bipolar tungsten electrodes placed into area CA3. The EPSP initial slope (mV/ms) was determined from the average waveform of four consecutive responses. Sp-cAMPS (50 μM; Biolog, La Jolla, CA), cycloheximide (60 μM; Sigma), or Rp-cAMPS (100 μM; Biolog) were bath applied for durations indicated in the figure legends. Four to six slices per condition were used for these experiments. In other experiments (antibody blocking), L-LTP was also induced by applying 4 trains of 100 Hz, 1 s tetanic stimulation separated by 5 min. The treatment of all animals was strictly in accordance with institutional and NIH guidelines.

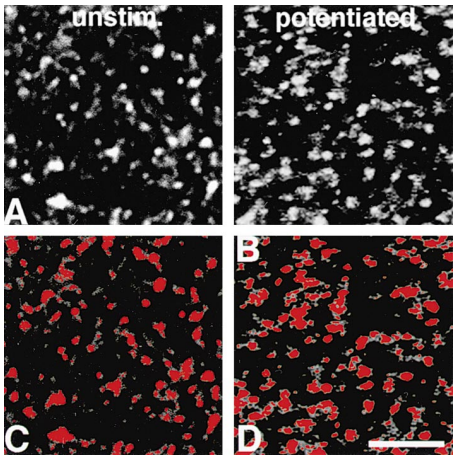

Figure 10. Thresholding Analysis of Immunoreactive Puncta

Single optical section of a control slice (A and C) or an Sp-cAMPS-potentiated slice (B and D) immunoreacted with N-cadherin and visualized with Oregon green. A sample series of images from different slices was used to establish optimal brightness and contrast levels. The images shown in (A) and (C) were imported into NIH Image; the density slice function was used to highlight individual puncta (shown in red in [B] and [D]). This was achieved by establishing a threshold that yielded the greatest number of individual puncta without causing a fusion of puncta.

Immunocytochemistry and Quantitative Analyses of Synaptic Puncta Number

Slices were removed from the recording chamber and fixed in 4% paraformaldehyde using brief microwave irradiation to enhance penetration of the fixative into the interior of the slice (Jensen and Harris, 1989). Frozen, 30 μm thick subsections were coimmunolabeled for synaptophysin (mouse, 1:10; Boeringher Mannheim Biochemicals) and N-cadherin using a previously characterized affinity-purified guinea pig N-cadherin antibody raised against the first extracellular (EC1) domain of N-cadherin (Fannon and Colman, 1996). Antibody binding was visualized immunofluorescently using two different fluorophores as described (Benson and Tanaka, 1998). Sections were imaged on a Zeiss 410 laser-scanning confocal microscope (LSM) using a 100×, 1.4 N.A. oil-immersion objective at a zoom of 4. Four to twenty single optical images from stratum radiatum of CA1 were taken from each section; 3–5 sections were analyzed per slice; and 3–12 slices were analyzed per condition (n values given in Results). Brightness and contrast were maintained within experiments but varied slightly across experiments to keep values within the maximum range of gray values (using maximum and minimum color indicators on sample images to set values). An investigator blinded to slice conditions obtained quantitative data using NIH Image (v. 1.61) in which a thresholding function (density slice) was employed to exclude from analysis all but immunolabeled puncta (Chen et al., 1995; Craig et al., 1996; Gomperts et al., 1998; Tanaka et al., 2000) (Figure 10). Thresholds were adjusted slightly

between sections to maximize numbers of puncta and minimize the extent of fusion between puncta, but the threshold values did not differ significantly within or across conditions (e.g., control value = 116.5 ± 5.3, Sp-cAMPS stimulated value = 116.9 ± 5.2, mean ± SEM, unpaired, two-tailed t test, $p = 0.96$). For each image, the cross-sectional area and intensity of individual puncta and total number of puncta was assessed. Data within sections were averaged and conditions were compared using Student's t test (two groups) or one-way ANOVA and a Fisher's post hoc test (three or more groups) with a level of significance of $p < 0.05$.

To determine the degree of N-cadherin and synaptophysin codistribution, N-cadherin and synaptophysin confocal images were overlaid, and the proportion of pixels that overlapped was determined using a colocalization macro (Zeiss LSM, Thornwood, NY). Data from unstimulated control and potentiated slices were compared using a two-tailed t test.

To control for Sp-cAMPS-induced changes in the area of the neuropil, unstimulated control slices ($n = 2$) and potentiated slices ($n = 2$) were processed immunofluorescently as described using anti-MAP2 (mouse, 1:500; Sigma) and anti-GFAP (rabbit, 1:50; Biomeda) antibodies. Single confocal images (4 per section) were taken through stratum radiatum of area CA1 and using image analysis software (Zeiss LSM, Thornwood, NY); the area that was occupied by immunolabeling was assessed after subtracting from the field unlabeled portions removed by a visually established threshold. Statistically significant differences between conditions were determined by Student's t test.

Immunoelectron Microscopy

Sp-cAMPs-potentiated ($n = 2$) and unstimulated control ($n = 3$) slices were fixed as described above with the addition of 0.1% glutaraldehyde. Thick sections were processed by a freeze-substitution, low-temperature Lowicryl embedding protocol for postembedding immunocytochemistry (van Lookeren Campagne et al., 1991; Chaudhry et al., 1995). After polymerization, ultra-thin sections were cut, mounted on nickel grids, and etched. N-cadherin antibody binding was visualized with 10 nm gold-conjugated secondary antibodies on a Jeol 1200 EX electron microscope.

Immunoblotting and Immunoprecipitation

Control and potentiated hippocampus slices (8 slices each) were extracted in 0.5 ml of lysis buffer (150 mM NaCl, 20 mM Tris-HCl [pH 7.5], 1% NP-40, 1% Triton X-100, Ca^{2+}-free, leupeptin 0.5 mg/ml, pefabloc 0.1 mol, aprotinin 1 mg/ml) for 30 min. After tissue lysis, the samples were centrifuged for 30 min at 15,000 rpm, and the supernatants were separated for immunobloting and immunoprecipitation. The Triton-insoluble fractions were treated with 50 μl of SDS sample buffer containing urea (70 mM Tris-NaOH [pH 6.8], 8 M urea, 2.5% SDS, 0.1 M DTT, 10% glycerol), and 5 μl of this mixture was used for gel electrophoresis followed by immunoblot. Protein concentrations were determined with the BCA protein assay (Pierce, Rockford, IL), and samples were run on 7.5% SDS–PAGE, transferred to nitrocellulose, blocked with 5% milk protein, and incubated overnight with rabbit anti-N-cadherin EC1 domain antibody. After secondary antibody incubation and routine washing, blots were developed with the ECL chemiluminescence system (NEN Life Sciences Products). Blots ($n = 6$) were scanned using identical parameters, and band intensity was assessed using an NIH Image (v. 1.61) macro for quantifying band intensity from gels. For each blot, band intensity from potentiated slices was normalized to that of unstimulated control slices. Data were compared using a paired Student's t test. For immunoprecipitation, the supernatants were incubated with guinea pig anti-N-cadherin EC1 domain antibody (30 μl per sample) for 2 hr at 4°C. The antigen-IgG complexes were precipitated by sequential incubations with protein A–Sepharose 4B. The immunoprecipitates were washed extensively and boiled with SDS sample buffer; the proteins in the immunoprecipitates were detected by immunoblotting ($n = 2$) with rabbit anti-N-cadherin EC1 domain antibody. Band intensity was quantified and compared as above.

Metabolic Labeling by ^{35}S-Methionine Incorporation

^{35}S-methionine was added (final concentration 3.4 mCi/ml) to 42 hippocampal slices maintained at 37°C in oxygenated Ringer's solution for 45 min. To one group of 14 slices, cyclohexamide (60 μM) was added; 0.5 hr after this, Sp-cAMPS (50 μM) was added to both the cyclohexamide group and to a second group of 14 slices. After a 15 min period of exposure to Sp-cAMPS, all slices were transferred to radioactive Ringer's solution. Seven slices were removed at 15 min and another 7, and the cyclohexamide group were removed at 30 min following Sp-cAMPS exposure. A third group of 14 slices served as unstimulated controls and was maintained in radioactivity for comparable periods. Slices were extracted as described above, with the exception that 1% SDS was added to the lysis buffer, and either run as a lysate directly on 7.5% SDS–PAGE or immunoprecipitated first as described above. The gel was dried and exposed to phosphorimager plates and analyzed using a phosphorimager (Storm, Molecular Dynamics). Band intensity was quantified using NIH image (see above). To account for differences in background across lanes, band intensity was normalized to lane intensity (intensity over an equal area).

N-Cadherin Antibody Blocking Experiments

Guinea pig N-cadherin antibody 1258 was generated against a purified EC1-EC2 domain of N-cadherin; guinea pig N-cadherin antibody 1260 was generated against an N-cadherin EC1 domain isolated and purified from a glutathione S-transferase (GST) EC1 fusion protein. Antisera were generated by Covance and then purifed on N-cadherin (EC1) BrCN-activated Sepharose CL-4B. Specificity of antisera was confirmed by Western blot and by competition assay. P20 mouse brain extracts were subjected to 7.5% SDS–PAGE, transferred to nitrocellulose, and incubated with antibody diluted 1:10. Blots showed a single band of ~125 kDa that could be specifically competed out with purifed N-cadherin EC1 protein (10–20 μg), but not with BSA. An intracellular N-cadherin antibody was generated as a control—the affinity-purified rabbit polyclonal antibody was generated against a glutathione S-transferase fusion protein fused to the entire intracellular domain of mouse N-cadherin (Tanaka et al., 2000). Slices (3–6 per condition) were preincubated (1/100 dilutions) with either one of the two guinea pig anti-N-cadherin antibodies, preimmune guinea pig sera (derived from the animals producing the antibodies), or the intracellular N-cadherin control Ab for 3 hr and then transferred to the recording chamber.

L Cell Aggregation Assay

The preparation of mouse L cells stably transfected with N-cadherin and details of the adhesion assay were as described previously (Tamura et al., 1998). Briefly, cells were dissociated by 0.01% trypsin in the presence of 1 mM $CaCl_2$ and reduced to single-cell suspensions at densities of $1.4–2.3 \times 10^6$ cells/ml. Cells were allowed to aggregate for 30 min at 37°C with constant rotation in the presence of 4 mM Ca^{2+}. The degree of aggregation was calculated by the index $(N_0 - N_{30})/N_0$, where N_{30} is the total particle number after the 30 min incubation and N_0 is the total particle number at the initiation of incubation. N-cadherin antibody (Ab 1260, 1:100) or preimmune serum (1:100) was incubated with L cells for 30 min prior to the aggregation assay.

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References

Abel, T., Nguyen, P.V., Barad, M., Deuel, T.A., Kandel, E.R., and Bourtchouladze, R. (1997). Genetic demonstration of a role for PKA in the late phase of LTP and in hippocampus-based long-term memory. Cell *88*, 614–626.

Andersen, P., and Soleng, A.F. (1998). Long-term potentiation and spatial training are both associated with the generation of new excitatory synapses. Brain Res. Rev. *26*, 353–359.

Arndt, K., Nakagawa, S., Takeichi, M., and Redies, C. (1998). Cadherin-defined segments and parasagittal cell ribbons in the developing chicken cerebellum. Mol. Cell. Neurosci. *10*, 211–228.

Bahr, B.A., Staubli, U., Xiao, P., Chun, D., Ji, Z.X., Esteban, E.T., and Lynch, G. (1997). Arg-Gly-Asp-Ser-selective adhesion and the stabilization of long-term potentiation: pharmacological studies and the characterization of a candidate matrix receptor. J. Neurosci. *17*, 1320–1329.

Bailey, C.H., and Kandel, E.R. (1993). Structural changes accompanying memory storage. Annu. Rev. Physiol. *55*, 397–426.

Benson, D.L., and Tanaka, H. (1998). N-cadherin redistribution during synaptogenesis in hippocampal neurons. J. Neurosci. *18*, 6892–6904.

Bliss, T.V., and Collingridge, G.L. (1993). A synaptic model of memory: long-term potentiation in the hippocampus. Nature *361*, 31–39.

Blitzer, R.D., Wong, T., Nouranifar, R., Iyengar, R., and Landau, E.M. (1995). Postsynaptic cAMP pathway gates early LTP in hippocampal CA1 region. Neuron *15*, 1403–1414.

Bolshakov, V.Y., Golan, H., Kandel, E.R., and Siegelbaum, S.A. (1997). Recruitment of new sites of synaptic transmission during the cAMP-dependent late phase of LTP at CA3–CA1 synapses in the hippocampus. Neuron *19*, 635–651.

Brieher, W.M., Yap, A.S., and Gumbiner, B.M. (1996). Lateral dimerization is required for the homophilic binding activity of C-cadherin. J. Cell. Biol. *135*, 487–496.

Buchs, P.A., and Muller, D. (1996). Induction of long-term potentiation is associated with major ultrastructural changes of activated synapses. Proc. Natl. Acad. Sci. USA *93*, 8040–8045.

Calhoun, M.E., Jucker, M., Martin, L.J., Thinakaran, G., Price, D.L., and Mouton, P.R. (1996). Comparative evaluation of synaptophysin-based methods for quantification of synapses. J. Neurocytol. *25*, 821–828.

Casadio, A., Martin, K.C., Guistetto, M., Zhu, H., Chen, M., Bartsch, D., Bailey, C.H., and Kandel, E.R. (1999). A transient, neuron-wide form of CREB-mediated long-term facilitation can be stabilized at specific synapses by local protein synthesis. Cell *99*, 221–237.

Chang, F.-L., and Greenough, W.T. (1984). Transient and enduring morphological correlates of synaptic activity and efficacy change in the rat hippocampal slice. Brain Res. *309*, 35–46.

Chaudhry, F.A., Lehre, K.P., van Lookeren Campagne, M., Ottersen, O.P., Danbolt, N.C., and Storm-Mathisen, J. (1995). Glutamate transporters in glial plasma membranes: highly differentiated localizations revealed by quantitative ultrastructural immunocytochemistry. Neuron *15*, 711–720.

Chavez-Noriega, L.E., and Stevens, C.F. (1994). Increased transmitter release at excitatory synapses produced by direct activation of adenylate cyclase in rat hippocampal slices. J. Neurosci. *14*, 310–317.

Chen, K.S., Masliah, E., Mallory, M., and Gage, F.H. (1995). Synaptic loss in cognitively impaired aged rats is ameliorated by chronic human nerve growth factor infusion. Neuroscience *68*, 19–27.

Colman, D.R. (1997). Neurites, synapses, and cadherins reconciled. Mol. Cell. Neurosci. *10*, 1–6.

Covault, J., Liu, Q.Y., and El, D.S. (1991). Calcium-activated proteolysis of intracellular domains in the cell adhesion molecules NCAM and N-cadherin. Mol. Brain Res. *11*, 11–16.

Craig, A.M., Banker, G., Chang, W., McGrath, M.E., and Serpinskaya, A.S. (1996). Clustering of gephyrin at GABAergic but not glutamatergic synapses in cultured rat hippocampal neurons. J. Neurosci. *16*, 3166–3177.

Einheber, S., Schnapp, L.M., Salzer, J.L., Cappiello, Z.B., and Milner, T.A. (1996). Regional and ultrastructural distribution of the alpha 8 integrin subunit in developing and adult rat brain suggests a role in synaptic function. J. Comp. Neurol. *370*, 105–134.

Engert, F., and Bonhoeffer, T. (1999). Dendritic spine changes associated with hippocampal long-term synaptic plasticity. Nature *399*, 66–70.

Fannon, A., and Colman, D.R. (1996). A model for central synaptic junctional complex formation based on the differential adhesive specificities of the cadherins. Neuron *17*, 423–434.

Fields, R.D., and Itoh, K. (1996). Neural cell adhesion molecules in activity-dependent development and synaptic plasticity. Trends Neurosci. *19*, 473–480.

Fischer, M., Kaech, S., Knutti, D., and Matus, A. (1998). Rapid actin-based plasticity in dendritic spines. Neuron *20*, 847–854.

Fletcher, T.L., Cameron, P., De Camilli, P., and Banker, G. (1991). The distribution of synapsin I and synaptophysin in hippocampal neurons developing in culture. J. Neurosci. *11*, 1617–1626.

Frey, U., and Morris, R.G.M. (1998). Synaptic tagging: implications for late maintenance of hippocampal long-term potentiation. Trends Neurosci. *21*, 181–188.

Frey, U., Krug, M., Reymann, K.G., and Matties, H. (1988). Anisomycin, an inhibitor of protein synthesis, blocks late phases of LTP phenomena in the hippocampal CA1 region in vitro. Brain Res. *452*, 57–65.

Frey, U., Huang, Y.Y., and Kandel, E.R. (1993). Effects of cAMP simulate a late stage of LTP in hippocampal CA1 neurons. Science *260*, 1661–1664.

Geiger, B., Volberg, T., Ginsberg, D., Bitzur, S., Sabanay, I., and Hynes, R.O. (1990). Broad spectrum pan-cadherin antibodies, reactive with the C-terminal 24 amino acid residues of N-cadherin. J. Cell Sci. *97*, 607–614.

Geinisman, Y., de Toledo-Morrell, L., Morrell, F., Heller, R.E., Rossi, M., and Parshall, R.F. (1993). Structural synaptic correlate of long-term potentiation: formation of axospinous synapses with multiple, completely partitioned transmission zones. Hippocampus *3*, 435–445.

Gomperts, S.N., Rao, A., Craig, A.M., Malenka, R.C., and Nicoll, R.A. (1998). Postsynaptically silent synapses in single neuron cultures. Neuron *21*, 1443–1451.

Halpain, S., Hipolito, A., and Saffer, L. (1998). Regulation of F-actin stability in dendritic spines by glutamate receptors and calcineurin. J. Neurosci. *18*, 9835–9844.

Huang, Y.Y., Nguyen, P.V., Abel, T., and Kandel, E.R. (1996). Long lasting forms of synaptic potentiation in the mamalian hippocampus. Learn. Mem. *3*, 74–85.

Huntley, G.W., and Benson, D.L. (1999). Neural (N)-cadherin at developing thalamocortical synapses provides an adhesion mechanism for the formation of somatopically organized connections. J. Comp. Neurol. *407*, 453–471.

Impey, S., Mark, M., Villacres, E.C., Poser, S., Chavkin, C., and Storm, D.R. (1996). Induction of CRE-mediated gene expression by stimuli that generate long-lasting LTP in area CA1 of the hippocampus. Neuron *16*, 973–982.

Inoue, A., and Sanes, J.R. (1997). Lamina-specific connectivity in the brain: regulation by N-cadherin, neurotrophins, and glycoconjugates. Science *276*, 1428–1431.

Isaac, J.T., Nicoll, R.A., and Malenka, R.C. (1995). Evidence for silent synapses: implication for the expression of LTP. Neuron *15*, 427–434.

Jensen, F.E., and Harris, K.M. (1989). Preservation of neuronal ultrastructure in hippocampal slices using rapid microwave-enhanced fixation. J. Neurosci. Methods *29*, 217–230.

Kirov, S.A., and Harris, K.M. (1999). Dendrites are more spiny on mature hippocampal neurons when synapses are inactivated. Nat. Neurosci. *2*, 878–883.

Kirov, S.A., Sorra, K.E., and Harris, K.M. (1999). Slices have more synapses than perfusion-fixed hippocampus from both young and mature rats. J. Neurosci. *19*, 2876–2886.

Krucker, T., Siggins, G.R., and Halpain, S. (2000). Dynamic actin filaments are required for stable long-term potentiation (LTP) in area CA1 of the hippocampus. Proc. Natl. Acad. Sci. USA, in press.

Liao, D., Jones, A., and Malinow, R. (1992). Direct measurement of quantal changes underlying long-term potentiation in CA1 hippocampus. Neuron *9*, 1089–1097.

Liao, D., Hessler, N.A., and Malinow, R. (1995). Activation of postsynaptically silent synapses during pairing-induced LTP in CA1 region of hippocampal slice. Nature *375*, 400–404.

Linnemann, D., Gaardsvoll, H., Dalseg, A.-M., Zhernosekov, D., Lundgren, T., Edvardsen, K., and Bock, E. (1994). Characterization of N-cadherin messenger RNA and polypeptide expression in rat. Int. J. Dev. Neurosci. *12*, 441–450.

Liu, G., Choi, S., and Tsien, R.W. (1999). Variability of neurotransmitter concentration and nonsaturation of postsynaptic AMPA receptors at synapses in hippocampal cultures and slices. Neuron *22*, 395–409.

Luthl, A., Laurent, J.P., Figurov, A., Muller, D., and Schachner, M. (1994). Hippocampal long-term potentiation and neural cell adhesion molecules L1 and NCAM. Nature *372*, 777–779.

Ma, L., Zablow, L., Kandel, E.R., and Siegelbaum, S.A. (1999). Cyclic AMP induces functional presynaptic boutons in hippocampal CA3–CA1 neuronal cultures. Nat. Neurosci. *2*, 24–30.

Malenka, R.C., and Nicoll, R.A. (1999). Long-term potentiation—a decade of progress? Science *285*, 1870–1874.

Maletic-Savatic, M., Malinow, R., and Svoboda, K. (1999). Rapid dendritic morphogenesis in CA1 hippocampal dendrites induced by synaptic activity. Science *283*, 1923–1927.

Manabe, T., Togashi, H., Uchida, N., Suzuki, S.C., Hayakawa, Y., Yamamoto, M., Yoda, H., Miyakawa, T., Takeichi, M., and Chisaka, O. (2000). Loss of cadherin-11 adhesion receptor enhances plastic changes in hippocampal synapses and modifies behavioral responses. Mol. Cell. Neurosci. *15*, 534–546.

Martin, K.C., Casadio, A., Zhu, H., Yaping, E., Rose, J.C., Chen, M., Bailey, C.H., and Kandel, E.R. (1997). Synapse-specific, long-term facilitation of aplysia sensory to motor synapses: a function for local protein synthesis in memory storage. Cell *91*, 927–938.

Mayford, M., Barzilai, A., Keller, F., Schacher, S., and Kandel, E.R. (1992). Modulation of an NCAM-related adhesion molecule with long-term synaptic plasticity in Aplysia. Science *256*, 638–644.

Muller, D., Wang, C., Skibo, G., Toni, N., Cremer, H., Calaora, V., Rougon, G., and Kiss, J.Z. (1996). PSA-NCAM is required for activity-induced synaptic plasticity. Neuron *17*, 413–422.

Navone, F., Di Gioia, G., Jahn, R., Browning, M., Greengard, P., and De Camilli, P. (1989). Microvesicles of the neurohypophysis are biochemically related to small synaptic vesicles of presynaptic nerve terminals. J. Cell. Biol. *109*, 3425–3433.

Nguyen, P.V., Abel, T., and Kandel, E.R. (1994). Requirement of a critical period of transcription for induction of a late phase of LTP. Science *265*, 1104–1107.

Persohn, E., Pollerberg, G.E., and Schachner, M. (1989). Immunoelectron-microscopic localization of the 180 kD component of the neural cell adhesion molecule N-CAM in postsynaptic membranes. J. Comp. Neurol. *288*, 92–100.

Petralia, R.S., Esteban, J.A., Wang, Y.X., Partridge, J.G., Zhao, H.M., Wenthold, R.J., and Malinow, R. (1999). Selective acquisition of AMPA receptors over postnatal development suggests a molecular basis for silent synapses. Nat. Neurosci. *2*, 31–36.

Pokutta, S., Herrenknecht, K., Kemler, R., and Engel, J. (1994). Conformational changes of the recombinant extracellular domain of E-cadherin upon calcium binding. Eur. J. Biochem. *223*, 1019–1026.

Qi, M., Zhuo, M., Skalhegg, B.S., Brandon, E.P., Kandel, E.R., McKnight, G.S., and Idzerda, R.L. (1996). Impaired hippocampal plasticity in mice lacking the C^{β}_{1} catalytic subunit of cAMP-dependent protein kinase. Proc. Natl. Acad. Sci. USA *93*, 1571–1576.

Ryan, T.A., Reuter, H., Wendland, B., Schweizer, F.E., Tsien, R.W., and Smith, S.J. (1993). The kinetics of synaptic vesicle recycling measured at single presynaptic boutons. Neuron *11*, 713–724.

Schikorski, T., and Stevens, C.F. (1997). Quantitative ultrastructural analysis of hippocampal excitatory synapses. J. Neurosci. *17*, 5858–5867.

Schuster, C.M., Davis, G.W., Fetter, R.D., and Goodman, C.S. (1996). Genetic dissection of structural and functional components of synaptic plasticity. II. Fasciclin II controls presynaptic structural plasticity. Neuron *17*, 655–667.

Segal, M. (1995). Morphological alterations in dendritic spines of rat hippocampal neurons exposed to N-methyl-D-aspartate. Neurosci. Lett. *193*, 73–76.

Shapiro, L., and Colman, D.R. (1999). The diversity of cadherins and implications for a synaptic adhesive code in the CNS. Neuron *23*, 427–430.

Shepherd, G.M.G., and Harris, K.M. (1998). Three-dimensional structure and composition of CA3 → CA1 axons in rat hippocampal slices: implications for presynaptic connectivity and compartmentalization. J. Neurosci. *18*, 8300–8310.

Shi, S.-H., Hayashi, Y., Petralia, R.S., Zaman, S.H., Wenthold, R.J., Svoboda, K., and Malinow, R. (1999). Rapid spine delivery and redistribution of AMPA receptors after synaptic NMDA receptor activation. Science *284*, 1811–1816.

Shirayoshi, Y., Hatta, K., Hosoda, M., Tsunasawa, S., Sakiyama, F., and Takeichi, M. (1986). Cadherin cell adhesion molecules with distinct binding specificities share a common structure. EMBO J. *5*, 2485–2488.

Song, J.Y., Ichtchenko, K., Sudhof, T.C., and Brose, N. (1999). Neuroligin 1 is a postsynaptic cell-adhesion molecule of excitatory synapses. Proc. Natl. Acad. Sci. USA *96*, 1100–1105.

Sorra, K.E., and Harris, K.M. (1998). Stability in synapse number and size at 2 hr after long-term potentiation in hippocampal area CA1. J. Neurosci. *18*, 658–671.

Sorra, K.E., Fiala, J.C., and Harris, K.M. (1998). Critical assessment of the involvement of perforations, spinules, and spine branching in hippocampal synapse formation. J. Comp. Neurol. *398*, 225–240.

Squire, L.R., and Zola, S.M. (1997). Amnesia, memory and brain systems. Phil. Trans. R. Soc. Lond. B *353*, 1663–1673.

Staubli, U., Vanderklish, P., and Lynch, G. (1990). An inhibitor of integrin receptors blocks long-term potentiation. Behav. Neural Biol. *53*, 1–5.

Takeichi, M. (1990). Cadherins: a molecular family important in selective cell-cell adhesion. Annu. Rev. Biochem. *59*, 237–252.

Tamura, K., Shan, W.S., Hendrickson, W.A., Colman, D.R., and Shapiro, L. (1998). Structure-function analysis of cell adhesion by neural (N-) cadherin. Neuron *20*, 1153–1163.

Tanaka, H., Shan, W., Phillips, G.R., Arndt, K., Bozdagi, O., Shapiro, L., Huntley, G.W., Benson, D.L., and Colman, D.R. (2000). Molecular modification of N-cadherin in response to synaptic activity. Neuron *25*, 93–107.

Tang, L., Hung, C.P., and Schuman, E.M. (1998). A role for the cadherin family of cell adhesion molecules in hippocampal long-term potentiation. Neuron *20*, 1165–1175.

Toni, N., Buchs, P.A., Nikonenko, I., Bron, C.R., and Muller, D. (1999). LTP promotes formation of multiple spine synapses between a single axon terminal and a dendrite. Nature *402*, 421–425.

Uchida, H., Honjo, Y., Johnson, K.R., Wheelock, M.J., and Takeichi, M. (1996). The catenin/cadherin adhesion system is localized in synaptic junctions bordering transmitter release zones. J. Cell. Biol. *135*, 767–779.

van Lookeren Campagne, M., Oestreicher, A.B., van der Krift, T.P., Gispen, W.H., and Verkleij, A.J. (1991). Freeze-substitution and Lowicryl HM20 embedding of fixed rat brain: suitability for immunogold ultrastructural localization of neural antigens. J. Histochem. Cytochem. *39*, 1267–1279.

Vaughn, J.E. (1989). Fine structure of synaptogenesis in the vertebrate central nervous sytem. Synapse *3*, 255–285.

Woolley, C.S., Wenzel, H.J., and Schwartzkroin, P.A. (1996). Estradiol increases the frequency of multiple synapse boutons in the hippocampal CA1 region of the adult female rat. J. Comp. Neurol. *373*, 108–117.

Yamagata, M., Herman, J.P., and Sanes, J.R. (1995). Lamina-specific expression of adhesion molecules in developing chick optic tectum. J. Neurosci. *15*, 4556–4571.

Yap, A.S., Brieher, W.M., and Gumbiner, B. (1997). Molecular and functional analysis of cadherin-based adherens junctions. Annu. Rev. Cell Dev. Biol. *13*, 119–146.

Yoshida, C., and Takeichi, M. (1982). Teratocarcinoma cell adhesion: identification of a cell-surface protein involved in calcium-dependent cell aggregation. Cell *28*, 217–224.